

Ultrasensitive Time-Resolved Immunofluorometric Assay of Prostate-Specific Antigen in Serum and Preliminary Clinical Studies

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We developed an ultrasensitive method for measuring prostate-specific antigen (PSA) in serum. The assay includes a capture monoclonal anti-PSA antibody coated to microtiter wells, a biotinylated rabbit polyclonal detection antibody, and alkaline phosphatase (ALP)-labeled streptavidin. The activity of ALP is measured with the substrate difluoridyl phosphate; the released difluoridyl forms highly fluorescent complexes with Tb³⁺-EDTA that are quantified with microsecond time-resolved fluorometry. The assay is precise and accurate and correlates well with the established Hybritech Tandem™-PSA kit. Its distinguishing feature is extreme sensitivity (lowest limit of detection is 0.002 µg/L or 2 × 10⁶ PSA molecules per assay). This is the most sensitive PSA assay reported thus far; we used it to quantify PSA in patients who had undergone radical prostatectomy. Many patients had <0.01 µg/L PSA in their serum. This method could have important clinical applications in postsurgical early detection of relapse or residual prostate cancer, as recently suggested in the literature (Clin Chem 1992;38:1930-2).

Indexing Terms: tumor markers · terbium chelates · alkaline phosphatase conjugate · biotin-streptavidin interaction

Prostate-specific antigen (PSA) is a 30-kDa kallikrein-like protease found in the prostate and in seminal plasma (1-4).² PSA is responsible for the liquefaction of the clot formed immediately after ejaculation (5, 6). In 1980, PSA concentrations were first reported to be increased in prostate cancer patients (7), and currently tests for PSA are widely used to monitor patients with prostate cancer (8-12). The PSA assay has been proposed as a screening test for prostatic carcinoma, in combination with rectal examination (13, 14).

The methodology for measuring PSA in serum is based on immunological assays with monoclonal and (or) polyclonal antibodies. A competitive-type assay is commercially available and was recently modified to improve sensitivity (15). Many other commercially available assays are of the noncompetitive type and incorporate radioactive nuclides or enzymes as labels.

Fully automated assays have been introduced recently and their performance evaluated (16, 17). A time-resolved immunofluorometric procedure that uses Eu³⁺ as label has also been published (18). Clinical and analytical aspects of the PSA test have recently been reviewed (8, 9, 19). Another prostate-specific protein, prostate secretory protein, has also been proposed as a useful marker for monitoring patients with prostate cancer (20).

PSA assays are relatively difficult to develop for many reasons. For example, PSA is present in serum in various forms, some of which are not well-characterized. Stenman et al. (21) and Lilja et al. (22) found that PSA is present in serum predominantly as a complex with α₁-antichymotrypsin (ACT) and to a lesser extent with other serum proteins such as α₂-macroglobulin and α₁-antitrypsin inhibitor. Another PSA fraction is free, but it is not clear whether this is a PSA zymogen, an inactive molecule identified in seminal fluid (23), or active PSA. Commercially available kits for PSA quantify mainly the free and ACT-bound PSA.

Another difficulty associated with PSA assays is the lack of a universally acceptable standard preparation (24). The current commercial kit standards are based on human serum or synthetic matrices with bovine serum albumin (BSA) and contain added PSA isolated from human seminal plasma. Thus, the various PSA subfractions in the standards are potentially different from those in the serum. These differences may lead to different patients' results because monoclonal and (or) polyclonal antibodies recognize the PSA subfractions with different affinities (21, 22). One way to minimize the problem is to adjust the assays to match the results of the Hybritech (San Diego, CA) Tandem™-PSA kit, which is widely used and established in the field (16, 19). However, good correlation between assays does not necessarily mean that the same PSA subfractions are measured, because the correlation between free and bound PSA is reasonably good (21).

Graves (19) and Vessella et al. (16) stressed the importance of new highly sensitive PSA assays that can be used to monitor prostate cancer patients after radical prostatectomy. Such assays, which should be capable of detecting at least 0.1 µg/L PSA, have already proved useful for detecting early relapse (15, 16, 19). Currently, the most sensitive PSA assay (16, 17) has an analytical lowest limit of detection of 0.03 µg/L and a biological detection limit of 0.06-0.1 µg/L. Here we describe the development of a new PSA assay based on the use of monoclonal and polyclonal antibodies and enzymatically amplified time-resolved immunofluorometry with terbium chelates. The method correlates well with the

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² Nonstandard abbreviations: PSA, prostate-specific antigen; BSA, bovine serum albumin; GARIG-ALP, alkaline phosphatase-conjugated goat anti-rabbit IgG; DFP, difluoridyl phosphate; ACT, α₁-antichymotrypsin; and SA-ALP, alkaline phosphatase-conjugated streptavidin.

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established Hybritech assay. The analytical limit of detection is 0.002 $\mu\text{g/L}$, the lowest reported for this analyte. With this assay, we were able to quantify PSA concentrations in serum of patients after prostatectomy.

Materials and Methods

Instrumentation. For measuring liquid-phase Tb^{3+} fluorescence in white microtiter wells, we used the Cyberfluor (Toronto, Canada) 615™ Immunoanalyzer, a time-resolved fluorometer. The time-gate settings of the instrument and the interference filter in the emission pathway have been described elsewhere (25, 26).

Reagents and solutions. All reagents were purchased from Sigma Chemical Co., St. Louis, MO, unless otherwise stated. The coating solution was 50 mmol/L Tris buffer, pH 7.80, containing 0.5 g of sodium azide per liter. The wash solution was 5 mmol/L Tris buffer, pH 7.80, containing 0.15 mol of NaCl and 0.5 g of polyoxyethylenesorbitan monolaurate (Tween 20) per liter. The substrate buffer was 0.1 mol/L Tris buffer, pH 9.1, containing 0.15 mol of NaCl, 1 mmol of MgCl_2 , and 0.5 g of sodium azide per liter. The substrate stock solution was a 10 mmol/L difunilal phosphate (DFP) solution in 0.1 mol/L NaOH, available from Cyberfluor. The developing solution contained 1 mol of Tris base, 0.4 mol of NaOH, 2 mmol of TbCl_3 , and 3 mmol of EDTA per liter (no pH adjustment). This solution was prepared as described previously (23, 24) and is commercially available from Cyberfluor. The assay buffer was 50 mmol/L Tris buffer, pH 7.80, containing 60 g of BSA, 0.5 mol of KCl, 0.5 g of sodium azide, 50 mL of normal mouse serum, and 5 g of Triton X-100 per liter. The diluent for the polyclonal biotinylated detection antibody and alkaline phosphatase-conjugated streptavidin (SA-ALP) was 50 mmol/L Tris buffer, pH 7.80, containing 60 g of BSA per liter. The diluent for alkaline phosphatase-conjugated goat anti-rabbit IgG (GARlg-ALP) was the same as for the polyclonal biotinylated detection antibody but also contained 40 mL/L goat serum. The blocking solution was 50 mmol/L Tris buffer, pH 7.80, containing 10 g of BSA per liter.

Antibodies. The mouse monoclonal (MBPO405) and the rabbit polyclonal (PBP0101) anti-PSA antibodies were purchased from Medix Biotech, Foster City, CA. The SA-ALP conjugate was purchased from Jackson ImmunoResearch, West Grove, PA, as was the affinity purified, Fc fragment-specific GARlg-ALP. A polyclonal rabbit antibody against ACT was purchased from Dakopatts (Glostrup, Denmark).

Standards. Because of the unavailability of a universally accepted standard for PSA, we used PSA standards in 50 mmol/L Tris buffer, pH 7.80, containing 60 g/L BSA. A stock PSA solution containing PSA purified from human seminal plasma was purchased from Scripps Laboratories, San Diego, CA. Our final standard solutions were calibrated against standards from the Hybritech Tandem-PSA kit. For routine use we recommend six PSA standards, at concentrations 0, 0.025, 0.1, 0.5, 2, and 10 $\mu\text{g/L}$. These are stable for at least 1 month at 4 °C.

Comparison method and patients' samples. For comparison we used the Tandem-PSA enzyme immunoassay kit from Hybritech. Patients' samples analyzed by the Hybritech assay were stored at -70 °C for ≤ 1 month.

Biotinylation of polyclonal anti-PSA antibody. The polyclonal anti-PSA antibody, purified by ion-exchange chromatography, was dialyzed overnight against 5 L of a 0.1 mol/L sodium bicarbonate solution. This stock antibody solution ($\sim 2\text{ g/L}$) was diluted twofold with 0.5 mol/L carbonate buffer, pH 9.1. To this solution we added 1 mg of the *N*-hydroxysuccinimide ester of biotin (NHS-LC-Biotin; Pierce Chemical Co., Rockford, IL) dissolved in 50 μL of dimethyl sulfoxide and incubated it for 2 h at room temperature with continuous stirring. This biotinylated antibody was used without further purification and could be stored without loss at 4 °C for at least 6 months.

Coating of microtiter wells. White, opaque 12-well microtiter polystyrene strips were obtained from Dynatech Laboratories, Alexandria, VA. The wells were coated overnight at room temperature with monoclonal anti-PSA antibody in the coating buffer, 5 mg/L. Before use, the wells were washed twice and blocked for 1 h with 200 μL per well of the blocking solution.

Assay procedure. Wash the strips six times. Into each well pipet 50 μL of serum samples or PSA standards and add 50 μL of assay buffer per well. Incubate for 3 h at room temperature with continuous mechanical shaking; then wash six times. Add 100 μL per well of the biotinylated rabbit polyclonal detection antibody, diluted 1000-fold in the polyclonal detection antibody diluent (100 ng of antibody per well). Incubate for 1 h as above and then wash six times. Add 100 μL per well of SA-ALP conjugate, diluted 30 000-fold in the SA-ALP diluent (3 ng of conjugate per well). Incubate for 15 min as above and then wash six times. Add 100 μL per well of the DFP substrate, diluted 10-fold just before use in the substrate buffer (working DFP substrate solution, 1 mmol/L) and incubate for 10 min at room temperature with shaking. Add 100 μL per well of the developing solution, mix by shaking for 1 min, and read the Tb^{3+} -specific fluorescence with the Cyberfluor 615 Immunoanalyzer. Data reduction is performed automatically.

Assay of the PSA-ACT complex. This assay is the same as the PSA assay described above but, instead of using the biotinylated polyclonal rabbit anti-PSA antibody, we used the polyclonal rabbit ACT antibody, diluted 500-fold in the SA-ALP conjugate diluent. We then added 100 μL of 5000-fold-diluted GARlg-ALP conjugate (20 ng per well) and incubated for 30 min with shaking. After washing the wells six times, we completed the assay by adding the DFP substrate as described in the PSA assay. No effort was made to calibrate this assay because of the unavailability of standard PSA-ACT complex.

HPLC. HPLC was performed with a Shimadzu system with an absorbance monitor at 280 nm (Shimadzu Corp., Kyoto, Japan). The mobile phase was a 0.1 mol/L Na_2SO_4 -0.1 mol/L NaH_2PO_4 solution, pH 6.80. The flow

rate was 0.3 mL/min and the HPLC was run isocratically. The gel-filtration column was a Bio-Sil SEC-250 column, 600 × 7.5 mm (Bio-Rad Labs., Richmond, CA). The column was calibrated with a molecular mass standard solution from Bio-Rad, containing thyroglobulin (670 kDa), IgG (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and cyanocobalamin (1.4 kDa). HPLC fractions (0.3 mL) were collected with a fraction collector (Model FRAC-100; Pharmacia, Uppsala, Sweden).

Results

Assay optimization. To attain the highest possible sensitivity, during the initial stages of assay development we considered various combinations of commercially available monoclonal and polyclonal antibodies. The pair we selected gave the most sensitive and accurate results. All diluents were optimized for protein, salt, goat or mouse serum, and detergent presence and concentration, to obtain the highest possible signal and the lowest possible background, and at the same time eliminate or minimize the effect of heterophile antibodies. Human anti-goat antibodies, present in some patients' sera and interfering with earlier versions of this assay, were effectively eliminated with use of the biotin-streptavidin system and the reagents described. Likewise, all antibody amounts were carefully optimized, along with the incubation times and temperature (25 °C vs 37 °C). Although it was possible to perform the assay by incubating the monoclonal and polyclonal antibodies with the sample in one step, we did not because this reduced the sensitivity ~5–10-fold, and we also wanted to avoid the high-dose hook effect. We tried to directly conjugate the polyclonal antibody to ALP, but this reduced the sensitivity 5–10-fold in comparison to our final optimized assay.

Calibration curves. Representative calibration curves for the proposed assay are shown in Figure 1. With 50- μ L sample volumes, the curve is useful for PSA quantification up to 10 μ g/L. We also used sample volumes of 25, 10, and 5 μ L and extended the assay range to 25, 50, and 100 μ g/L, respectively, without changing any other assay conditions. All other data reported in this paper were generated with 50- μ L sample volumes.

Lowest limit of detection. The lowest limit of detection, defined as the concentration of PSA that could be distinguished from zero with 95% confidence, was 0.002 μ g/L. This value was established by running replicates of the zero standard and a standard with a very low concentration of PSA (0.0125 μ g/L) and calculating the PSA concentration that corresponds to the fluorescence of the zero standard plus 2 SD. This detection limit corresponds to 0.1 pg of PSA per assay, which is equivalent to $\sim 2 \times 10^6$ molecules. We have similarly calculated detection limits with sample volumes of 25, 10, and 5 μ L; these were 0.004, 0.007, and 0.014 μ g/L, respectively.

Vessella et al. (16) and Graves (19) proposed and endorsed, respectively, the calculation of the biological limit of detection of PSA assays. With their method, and a CV of 21.4% at a serum PSA concentration of 0.016

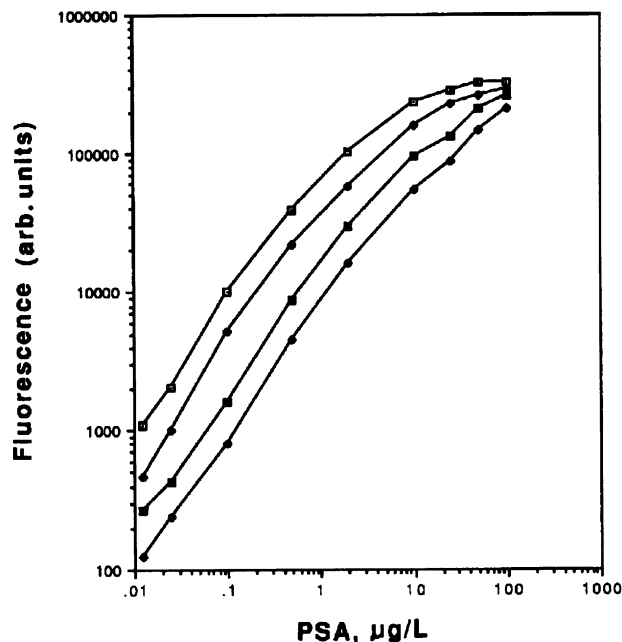


Fig. 1. Calibration curves of the proposed PSA assay with various sample volumes: 50 μ L (\square), 25 μ L (\blacklozenge), 10 μ L (\square), and 5 μ L (\diamond). Each point is the mean of three measurements. The CVs of the fluorescence readings were <5% in all cases. The fluorescence of the zero standard (1400–2000 arbitrary units) was subtracted from all other measurements

μ g/L, we calculated our biological limit of detection to be ~ 0.01 μ g/L.

Reproducibility. The reproducibility was checked at various PSA concentrations, particularly at low PSA concentrations, to establish the reliability of PSA quantification. The results are summarized in Table 1. CVs <14% in the whole measuring range could be achieved, except at a PSA concentration close to the biological limit of detection (CV 21.4%).

Analytical recovery. Recovery experiments were performed by adding a small aliquot (<10% of total sample volume) of a seminal plasma-based 100 μ g/L PSA standard to sera from females and males. The recovery ranged from 76% to 96% with a mean of 84%. The lower-than-expected recovery, also reported previously (16), is presumably due to PSA binding to serum proteins, forming complexes that are not measurable by the PSA assay (21). When the recovery experiments were

Table 1. Within-Run and Day-to-Day Precision of the PSA Assay

| Within-run ^a | | | Day-to-day ^b | | |
|-------------------------|--------------------------|-------|-------------------------|--------------------------|-------|
| Sample | Mean \pm SD, μ g/L | CV, % | Sample | Mean \pm SD, μ g/L | CV, % |
| 1 | 0.016 \pm 0.0034 | 21.4 | 8 | 0.072 \pm 0.0090 | 12.2 |
| 2 | 0.034 \pm 0.0045 | 13.2 | 9 | 0.20 \pm 0.029 | 14.5 |
| 3 | 0.066 \pm 0.0074 | 11.2 | 10 | 0.27 \pm 0.037 | 13.8 |
| 4 | 0.24 \pm 0.033 | 13.6 | 11 | 0.76 \pm 0.097 | 12.9 |
| 5 | 0.41 \pm 0.031 | 7.6 | 12 | 0.99 \pm 0.13 | 13.6 |
| 6 | 0.83 \pm 0.065 | 7.8 | 13 | 5.98 \pm 0.61 | 10.2 |
| 7 | 2.25 \pm 0.12 | 5.4 | | | |

^a n = 12 in all cases.

^b n = 12 runs over a period of 1 week.

repeated by adding PSA from the sera of two men with prostate cancer to sera from two women and one post-prostatectomy man, the recovery was close to the theoretically expected recovery (mean = 106% ± 7%, n = 12) (16).

Dilution linearity and interferences. We diluted three serum samples 2- to 128-fold with our zero standard (60 g/L BSA) and reanalyzed them. The results are shown in Figure 2. There is a near-linear relation between PSA concentrations and dilution. The assay was found to be free of any interference by hemolysis (hemoglobin ≤ 4 g/L), lipemia (triglycerides ≤ 12 mmol/L), and bilirubin (≤ 400 μmol/L).

Reference range. We analyzed PSA in the serum of 133 apparently healthy men (ages 17–68 years), and the results are shown in Table 2. PSA values were < 2.84 μg/L. Recent reports (18) found an upper limit of 2.88 μg/L. Based on a much larger male population, Hybritech suggests an upper limit of < 4.0 μg/L (13). Our study suggests an increase of PSA in serum with age, especially in the group > 50 years (Table 2). We also analyzed 78 samples from apparently healthy females. Seventy-three serum samples had values < 0.02 μg/L; in the remaining five, PSA concentrations were 0.035, 0.063, 0.089, 0.12, and 0.51 μg/L. These data are in close agreement with those recently reported (16).

Correlations. We compared our assay with the Hybritech Tandem-PSA kit; the results are shown in Figure 3. There is an excellent correlation and agreement of results between the two methods.

HPLC studies. It is now clear that PSA is present in serum predominantly as a complex with ACT and to a lesser extent as free or bound to other serum proteins (21, 22). We studied the nature of the immunoreactivity in our assay by analyzing PSA in fractions separated

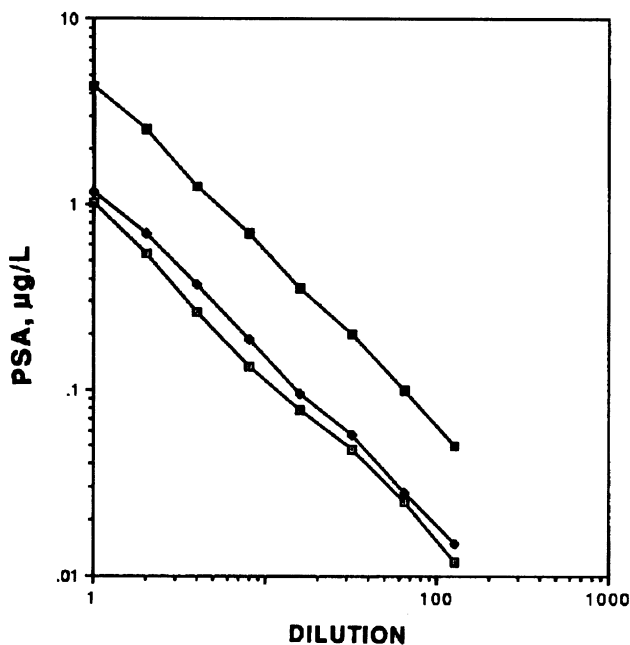


Fig. 2. Linearity studies
The three serum samples were diluted 2–128-fold with BSA (60 g/L)

Table 2. Serum PSA Concentrations from 133 Healthy Men

| Age, years | n | Conc, μg/L | | | |
|------------|----|-------------|--------|------|------|
| | | Mean ± SD | Median | Min. | Max. |
| <30 | 30 | 0.54 ± 0.44 | 0.41 | 0.12 | 1.86 |
| 30–49 | 69 | 0.58 ± 0.35 | 0.47 | 0.14 | 1.67 |
| 50–68 | 34 | 0.93 ± 0.65 | 0.67 | 0.15 | 2.84 |

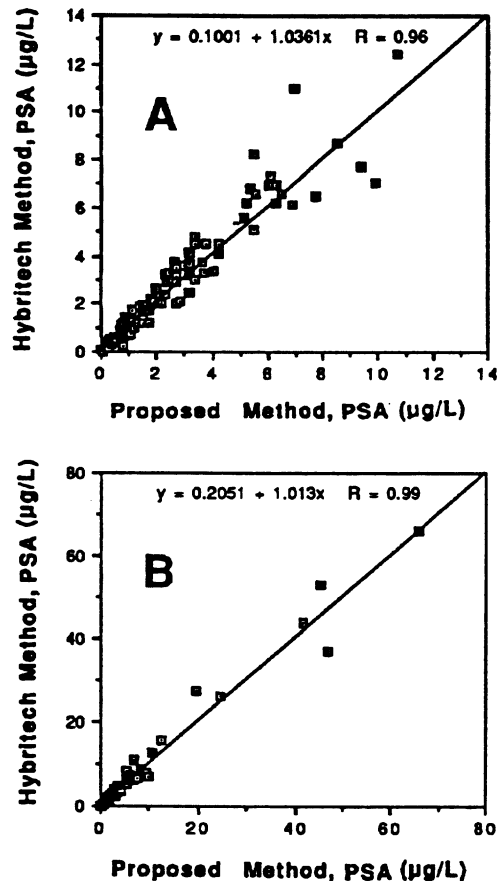


Fig. 3. Comparisons of the proposed PSA method and the Hybritech Tandem-PSA kit for PSA concentrations of 0–12 μg/L (A, n = 132) and 0–70 μg/L (B, n = 139)

All samples with PSA concentrations > 10 μg/L by the proposed method were analyzed after a 20-fold dilution with BSA (60 g/L, pH 7.80)

with HPLC by using a molecular sieve column. In these fractions we also analyzed specifically for the PSA-ACT complex by using a capture monoclonal anti-PSA antibody and a polyclonal rabbit anti-ACT antibody for detection. In serum, there are two major peaks of immunoreactivity, 100–110 kDa and 27–31 kDa. These two peaks represent the PSA-ACT complex, as verified with the PSA-ACT assay, and the free PSA (Figure 4). No immunoreactivity is detected with the PSA-ACT assay in the HPLC fractions from seminal plasma PSA, as expected.

A closer look at other minor fractions appearing in the chromatogram of the patient's serum (Figure 4, lower panel) reveals that there are another two and possibly three peaks of immunoreactivity, eluting at ~700–800 kDa (peak 1), 400 kDa (peak 2), and 250 kDa (peak 3). Peak 3 is recognized by the PSA-ACT assay and must

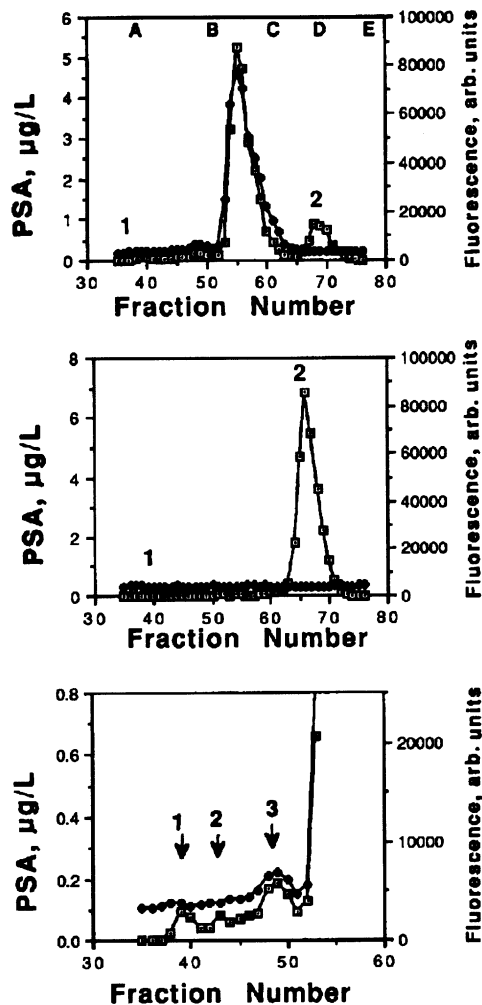


Fig. 4. Immunoreactivity measured in HPLC fractions with the PSA assay and the PSA-ACT assay

Upper panel: serum sample with PSA (87 $\mu\text{g/L}$) separated by HPLC and fractions analyzed by the PSA assay (\square , line 2) and the PSA-ACT assay (\blacklozenge , line 1). The first peak is recognized by both the PSA assay and the PSA-ACT assay and is due to the PSA-ACT complex. The second peak represents free PSA. Middle panel: seminal plasma PSA solution in BSA separated by HPLC and fractions analyzed by the PSA assay (\square , line 2) and the PSA-ACT assay (\blacklozenge , line 1). The one peak corresponds to free PSA. Lower panel: Detail of the chromatogram in upper panel of fractions 35–52. There are two (peaks 1, 3) and possibly a third minor peak (peak 2) eluting at 700–800 kDa (peak 1), 400 kDa (peak 2), and 250 kDa (peak 3). Peak 3 is also recognized by the PSA-ACT assay. For more details see text. A–E are molecular mass markers (kDa): 670 (A), 158 (B), 44 (C), 17 (D), and 1.4 (E). The fluorescence axis corresponds to the results for the PSA-ACT assay

represent another form of PSA-ACT complex, probably a dimer. Peak 1 most likely represents a complex of PSA with α_2 -macroglobulin (21).

The free PSA fraction is $\sim 20\%$ of total PSA in the chromatogram of serum in Figure 4. Another serum with total PSA of 1815 $\mu\text{g/L}$ was also studied; the free PSA was $<10\%$ of total.

Analysis of post-prostatectomy sera. We used our assay to analyze sera from 27 post-prostatectomy patients who are currently being monitored with the DPC IRMA-Count™ PSA kit (Diagnostic Products Corp., Los Angeles, CA). All samples had PSA values $<0.4 \mu\text{g/L}$ by the DPC kit. Our results are shown in Figure 5. Clearly, in 16 of these patients the PSA concentrations are sim-

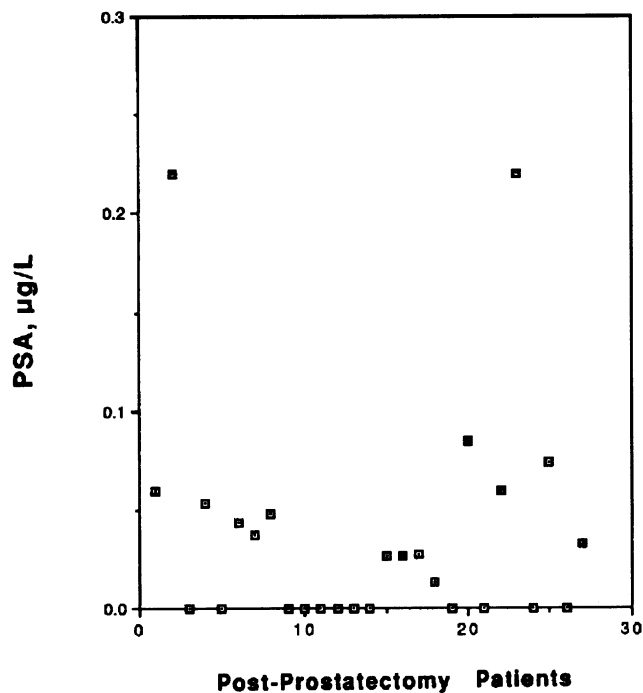


Fig. 5. Post-prostatectomy PSA concentrations in 27 patients

ilar to those found in women, i.e., $<0.02 \mu\text{g/L}$. In all but two patients the PSA concentrations were $<0.1 \mu\text{g/L}$, the amount currently considered the decision point for relapse. All patients are currently monitored by our assay to possibly detect relapse at an earlier stage.

Discussion

PSA assays are now performed routinely for the management of prostatic carcinoma. Although the same assay has also been proposed as a screening test for the early diagnosis of prostate cancer, in combination with rectal examination (13, 14), this issue remains controversial. Recent reports stress the importance of using ultrasensitive PSA assays that have the ability to quantify PSA at a concentration of at least $0.1 \mu\text{g/L}$ (15–17, 19). Such highly sensitive assays have already proven useful for the early detection of relapse, sometimes many months earlier than conventional PSA assays, which cannot quantify PSA at concentrations $<0.3 \mu\text{g/L}$ (15, 16). Another useful application of ultrasensitive PSA assays would be the early detection of residual disease after prostatectomy and the evaluation of the response to therapy. Patients with residual disease usually have PSA concentrations $>0.1 \mu\text{g/L}$ a few weeks after surgery. Patients with modestly increased concentrations of PSA after surgery (e.g., $0.5 \mu\text{g/L}$) show declining trends after radiotherapy; when therapy is successful, concentrations fall below $0.1 \mu\text{g/L}$ (16).

The most sensitive PSA assay reported is an enzyme immunoassay with fluorometric detection, automated on the IMx® analyzer (Abbott Diagnostics, Abbott Park, IL) (16, 17). The lowest limit of detection of this assay is $0.03 \mu\text{g/L}$. This assay was found to be precise, and PSA results $>0.06 \mu\text{g/L}$, which is considered the biological detection limit for this analyte, are easily quantifiable

(16). This assay has been criticized because of its limited capacity to run large numbers of samples and because it does not provide calibrators at concentrations $<2 \mu\text{g/L}$ (19).

We devised an assay based on enzymatically amplified time-resolved fluorometry with terbium chelates as labels, a methodology that has the potential for extreme sensitivity (25, 26). We selected a monoclonal/polyclonal antibody combination and optimized carefully all factors that affect assay and background signals, including composition of diluents, incubation times, amounts of reagents per assay, and temperature. Our optimized assay has an analytical detection limit of $0.002 \mu\text{g/L}$, and a biological detection limit of $0.01 \mu\text{g/L}$, about an order of magnitude lower than the IMx assay. Our assay has a working dynamic range $\leq 10 \mu\text{g/L}$, which can be extended to $100 \mu\text{g/L}$ by using smaller sample volumes, but at the expense of losing sensitivity. Because there is no international reference preparation for PSA, our assay was calibrated against the Hybritech Tandem-PSA kit, a widely used and FDA-approved method. There is an excellent correlation between our results and the Hybritech kit at PSA ranges $<12 \mu\text{g/L}$ and $\leq 70 \mu\text{g/L}$.

Our assay takes ~ 5 h to complete. However, because it is performed in microtiter wells and in a batch mode, many plates can be run in parallel. On the basis of our experience, and with use of automated pipetting and washing devices, 500–1000 samples could be completed in one working day. The microplate fluorometric reader quantifies fluorescence at a rate of 1 s/well and data reduction is automatic. In view of the high-volume testing anticipated or already existing for this analyte, our method may be an attractive candidate.

HPLC studies clearly demonstrated that our assay quantifies both the PSA-ACT complex and the free serum PSA. We verified that the PSA-ACT complex is the predominant form of PSA in serum. We were also able to detect a PSA-containing complex of very high molecular mass. We believe that this is the PSA- α_2 -macroglobulin complex, a form that until now was thought to be unmeasurable by PSA assays (21). Another PSA fraction not previously reported, of ~ 250 kDa, is also present in serum and is detected by both the PSA assay and an assay that detects the PSA-ACT complex. We speculate that this fraction is a dimer of the PSA-ACT complex.

Conventional PSA assays are widely used to monitor patients after radical prostatectomy. Usually, post-prostatectomy concentrations of PSA in serum fall below 0.3 – $0.4 \mu\text{g/L}$ a few weeks after surgery. Although it was postulated that post-prostatectomy patients should have PSA concentrations similar to those found in women, no assay was available that had sensitivity $<0.3 \mu\text{g/L}$ to establish the exact concentrations. Two reports, based on assays with biological detection limits of ~ 0.06 – $0.1 \mu\text{g/L}$, have clearly established that patients with radical prostatectomies should have PSA concentrations $<0.1 \mu\text{g/L}$ unless residual disease is present (16, 19). We here report preliminary evidence that many radical prostatectomy patients indeed have PSA concentrations similar to those found in most women, $<0.02 \mu\text{g/L}$. In-

terestingly, 12 of our 27 patients had PSA values $<0.01 \mu\text{g/L}$, which is the biological detection limit of our assay.

It is not clear whether patients with PSA concentrations $>0.02 \mu\text{g/L}$ by our assay have residual disease. Recent data (27, 28) have shown that PSA may be present in the periurethral glands of men. Possibly, these PSA-producing glands are removed to a variable degree during radical prostatectomy. More clinical studies are needed to establish whether the absolute PSA concentrations post-prostatectomy can be used to detect residual disease or whether progressively increasing PSA concentrations, even between 0.01 and $0.1 \mu\text{g/L}$, are more useful (29, 30). We are currently monitoring this group of 27 and other patients treated with radical prostatectomy to detect early relapse. At this point, we emphasize the importance of running quality-control sera in the ultrasensitive range to monitor assay performance and detect possible changes due to different batches of reagents or random variation. In our assay, the quality-control sera are in the PSA concentration range of 0.025 , 0.10 , and $0.30 \mu\text{g/L}$.

Although there is still no satisfactory treatment of cancer relapse, even if diagnosed early, our assay offers a biochemical means of tracking small numbers of cancer cells and their proliferation rate. Our assay, which is about an order of magnitude more sensitive than any other assay reported so far, may play an important role in cancer monitoring when improved treatments become available.

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